ABSTRACT

The alloimmune response is central to graft rejection. In the cellular response, recipient T cells recognize foreign antigen presented in a complex with major histocompatibility complex (MHC) proteins on the surface of specialized antigen-presenting cells. If the interaction between the T-cell receptor and the MHC complex is accompanied by appropriate costimulatory signals, then other signals and cytokines are triggered and the T cell proliferates and rejection ensues. Activated B cells produce antibodies that are graft specific. By targeting various steps in the recognition, proliferation, or activation phase of the alloimmune response, immunosuppressive agents alone or in combination can inhibit T-cell action and thereby reduce the risk of acute rejection. Because the alloimmune response also contributes to cardiac allograft vasculopathy, immunosuppressive agents may also limit this type of chronic rejection. Gene expression tests that measure the up- or downregulation of specific molecular and cell pathways are being developed as an alternative to the standard biopsy-based diagnosis of rejection. This review will discuss the alloimmune response, the mechanisms of immunosuppressive agents, and molecular diagnosis of graft rejection.

that bind to alloantigens (not just MHC antigens) in a way that focuses components of the innate immune system. In the classic complement pathway, complement binding to the antibodies and subsequent activation results in vascular injury and damage to the allograft. Activated macrophages and other immune cells may also bind to the antibodies, leading to antibody-mediated cell lysis. As discussed later, detection of complement or macrophages in the graft provides evidence of antibody-mediated rejection. Recall that the antibodies and the receptors found on the B and T cells, respectively, have highly variable regions that only become specific for antigen when the naïve cell is activated or mature.3

The T cells recognize alloantigens in 1 of 2 ways. In the direct pathway, the T-helper cell recognizes a foreign antigen on the graft cell itself; this foreign antigen is often associated with an MHC complex on the donor’s antigen-presenting cells (ie, an allogeneic antigen-presenting cell [APC]). In the indirect pathway, the foreign antigen is first broken down into small peptides by the recipient’s own APCs (ie, a self APC), such as dendritic cells, and these peptides are presented to naïve T cells in a complex with the recipient MHC antigens.2 In response, activated T-helper cells (CD4+) proliferate and produce cytokines that stimulate production of cytotoxic T cells (CD8+), B cells, and macrophages. All of these elements, spurred by the CD4+ T cell, cause destruction of the graft by direct lysis of target cells, antibody production, and delayed-type hypersensitivity reactions (Figure 1).4

The interactions between the MHC-peptide complex on the APCs and the receptors on the T cells are central to the complete alloimmune response.5 The interaction is often initiated by interdigitating dendritic cells, which are capable of recognizing potentially harmful foreign microorganisms or nonself constituents and can also be stimulated by endogenous activators, such as interferon α, heat-shock proteins, and tumor necrosis factor, released during infection. Dendritic cells break down the foreign antigen into smaller peptides and present these peptides in conjunction with MHC class II molecules at their cell surface. This presentation occurs in the secondary lymphoid tissues where T cells ordinarily circulate.

When the MHC complex is bound by receptors on the naïve T cell, both cells begin to express a variety of costimulatory molecules. These cell surface molecules provide the type of second, third, and fourth “hand-shakes” that are needed to ramp up the full immune response. This costimulation is provided, for example, by interactions of T cell CD28 with APC CD80/CD86 (also called B7) and also by T cell leukocyte function-associated antigen 1 with intercellular adhesion molecule 1. Such costimulatory signals are required for lymphocyte activation; without them, anergy or apoptosis will occur.6 Blocking interactions between the paired receptors involved in costimulation is a goal for many next-generation antirejection drugs.

Once a T cell is appropriately stimulated at the surface, a variety of events are prompted in the cytoplasm and nucleus. This downstream signaling involves several biochemical pathways and transcriptional activities that ultimately result in cell proliferation and differentiation. For example, 3 signal transduction pathways (calcium-calcineurin, mitogen-activated protein kinase, and protein kinase C-nuclear factor κ B) activate transcription factors that lead to the production of interleukin-2 (IL-2) receptor and IL-2 itself. This cytokine stimulates T-cell proliferation and, thus, via costimulation the T cell is able to fuel its own activation via generation of IL-2. Both IL-2 and IL-15 deliver growth signals through the phosphoinositide-3-kinase pathway and the molec-
ular target of rapamycin (mTOR) pathway, which initiates the cell cycle.\textsuperscript{7}

In attempting to clarify this complex and multi-step process, only a portion of which is covered here, some researchers have described 3 key signals required for cellular rejection. In this 3-step model, Signal 1 is the T-cell receptor binding to the MHC complex on the APC, Signal 2 consists of the costimulatory binding, and Signal 3 involves the binding of IL-2 to the IL-2 receptor.\textsuperscript{7}

The end result of this 3-step T-cell activation is all too clear to the transplant cardiologist. Without appropriate medical therapy, acute rejection can occur within days of transplantation (Figure 2).\textsuperscript{8} Antigen-experienced T cells infiltrate the graft and create endothelial inflammation (eg, tubulitis and endothelial arteritis), in addition to rejection lesions in the parenchyma or cardiac myocyte. This is mainly a cell-mediated immune response but antibodies may contribute as well. If acute rejection does not destroy the graft, adaptation occurs and can be stabilized by immunosuppressive drugs.

Although it also enlists elements of the alloimmune response, chronic rejection (Figure 2) also involves nonimmunologic mechanisms and is a fundamentally different pathophysiologic process.\textsuperscript{8} Ischemia-induced injury to the donor endothelium initiates a broad cascade of immunologic and inflammatory responses, including upregulation of complement, inflammatory mediators, chemoattractants, and cytokines. The end result, seen over a period of weeks and months, is vascular smooth muscle proliferation and the development of neointimal hyperplasia, matrix deposition, and lumen narrowing that is commonly known as chronic rejection of CAV.\textsuperscript{9,10}

**MECHANISMS OF IMMUNOSUPPRESSIVE DRUGS**

The primary goal of immunosuppression is to blunt the alloimmune response just described and thereby to prevent rejection. The targets of the main immunosuppressive agents are illustrated in Figure 3\textsuperscript{11,12} and can be summarized as follows:

- **Calcineurin inhibitors (eg, cyclosporine and tacrolimus):** Inhibit T-cell proliferation and activation via inhibition of calcineurin and, subsequently, IL-2 expression.
- **Antiproliferative agents (eg, azathioprine and mycophenolic acid):** Inhibit T-cell proliferation via inhibition of purine synthesis (necessary for DNA replication).
- **Corticosteroids:** Exert potent immunosuppressive and anti-inflammatory effects by altering transcription factors that control genes affecting growth factors, cytokines, and adhesion factors. Among the many synergistic and complementary effects of steroids, these agents inhibit production of IL-6 and, to some degree, IL-2.
- **Proliferation signal inhibitors (eg, sirolimus and everolimus):** Inhibit the mTOR after autocrine stimulation by IL-2 (thereby complementary to calcineurin inhibitors) to slow proliferation of T cells; these agents also inhibit proliferation of the
vascular smooth muscle cells typically seen in response to endothelial injury. Although sirolimus binds to the same protein as tacrolimus (FK binding protein-12), it does not inhibit calcineurin and the related IL-2 production; rather, it binds to mTOR, which blocks translation initiation and, ultimately, a variety of CD28 and IL-2 driven proliferation signals.13

- Induction therapy (e.g., anti–IL-2 receptor antibodies, anti-CD3 antibodies, and antithymocyte globulins): Intense perioperative immunosuppression usually involves use of agents such as: (1) basiliximab or daclizumab, which prevent IL-2 binding to the IL-2 receptor and thereby inhibit T-cell proliferation and activation; (2) anti-CD3 antibodies, such as murine monoclonal CD3 antibody, which blocks the T-cell receptor from binding to the antigen-presenting MHC antigens; and/or (3) antithymocyte globulin, which has an unknown mechanism but which inhibits a variety of T-cell receptors and proteins.7

Thus, the transplant specialist has a range of immunosuppressive tools that target, at varying levels and to different degrees, 2 of the 3 main signals involved in T-cell proliferation and activation. In terms of Signal 1, the anti-CD3 antibodies block MHC/peptide surface interactions with the T-cell receptor; the calcineurin inhibitors block the downstream actions of the stimulated CD3 receptor. Regarding Signal 3, the IL-2 receptor antibodies block the critical CD25 receptor on the cell surface while the mTOR inhibitors and antiproliferative agents interrupt specific steps in the chain of events following IL-2 stimulation.

Several agents, including an anti-CD154 monoclonal antibody and CTLA-4-Ig (an immunoglobulin fragment that blocks CD80/CD86, or B7), are in development to target Signal 2.4 The goal is that one or more of these investigational agents will eventually be shown to block the critical co-stimulation step and thereby elicit a prolonged state of T-cell quiescence even after foreign antigens are recognized in Signal 1.

Photopheresis is another method that is occasionally used in patients having severe rejections not responding to standard immunosuppressive therapies. This method involves removing white blood cells, treating them with methoxypsoralen, irradiating them with ultraviolet light, washing out the methoxypsoralen, and then returning the cells to the patient. Although the exact mechanism is unclear, this method appears to target proliferating cells and a randomized trial (N = 60) has documented a reduced mean number of rejections (0.91 vs 1.44, P = .04) at 6 months after transplant.14

UPDATE IN DIAGNOSING CELLULAR REJECTION

The most well-defined method for diagnosing cellular rejection still involves endomyocardial biopsy. This invasive surveillance involves 13 to 15 biopsies in the first year. Generally the sampling device is guided by echocardiogram or X ray through the internal jugular vein to the apex of the right ventricle. For a meaningful survey, each biopsy requires a minimum of 3 cardiac samples from 3 different sites. In recent years, as discussed in the following sections, a new system has been introduced to grade cardiac biopsies and molecular methods have been developed to reduce the need for invasive sampling and also, possibly, to improve early prediction of rejection events.
The endomyocardial biopsy is also used to diagnose antibody-mediated vascular rejection. Typically detected in the capillaries, this procedure often involves immunofluorescent staining of specimens for complement fragments C4d, C3c, and/or C1q. Another method is immunohistochemical staining for the CD68 antigen, which is associated with macrophages. Paraffin immunohistochemistry for C4d and standard hematoxylin and eosin staining of the capillaries are probably still the most common approaches. Deploying a combination of these techniques on the biopsy specimen will reveal antibody-related rejection activity, endothelial edema, and cellular infiltration; performed serially, the techniques will also show diminished staining as a rejection episode abates with treatment.15

**NEW PATHOLOGIC CRITERIA**

The old scoring system promulgated by the International Society for Heart and Lung Transplantation (ISHLT) classified the severity of rejection into 4 grades based on the extent of the lymphocyte or inflammatory infiltrate in the myocytes and also the extent of myocyte parenchymal damage.16 In this 1990 grading system, rejections graded as 0 or 1A or 1B would not be treated. The new system is based on the same criteria of infiltration and myocyte damage but combines several of the older classifications to create a simpler mild-moderate-severe grading of rejection (Table).16,17

In this new system, only grade 2R (moderate) and grade 3R (severe) require treatment; the old grade 2 specimen with focal infiltrate is now considered mild and not treated.

**NEW MOLECULAR METHODS**

In an attempt to produce less invasive methods for detecting rejection, a variety of gene expression tests on peripheral blood cells have been developed. Such tests, which measure the expression of select genes thought to be upregulated or downregulated during rejection, hold the potential for a more objective standard of classification and also—potentially—an earlier warning of impending rejection.

The development of one such expression test in the Cardiac Allograft Rejection Gene Expression Observational (CARGO) study illustrates the processes, assumptions, and clinical potential inherent in the new genomic-based assays. In CARGO, microarrays and then quantitative real-time polymerase chain reaction tests were used to identify genes that strongly correlated with the presence or absence of acute cellular rejection (Figure 4).18 The genes were selected and then validated based on comparison of expression results with biopsy results found in blinded pathology scoring in the same patient. Eventually, the hundreds of genes initially evaluated in early development were winnowed to the more practical number of approximately 20 in the current version of the diagnostic test. These selected genes are thought to play a role in a variety of pathways involved in response to the allograft—including T-cell or macrophage activation, T-cell migration, platelet activation, inflammation, steroid responsiveness, and hematopoiesis.18

Results from the CARGO study to date indicate that the test successfully distinguishes grade 3A or greater (moderate-to-severe) rejection from grade 0 rejection (P < .0001) and also from grades 1A or 2 (mild) rejection (P < .05; 1990 grading system).18,19 Significantly, if a patient maintains a low gene expres-

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**Table. Grading of Acute Cellular Rejection in Cardiac Biopsies: Criteria for Classification with Comparison of Old and New Grading Systems**

<table>
<thead>
<tr>
<th>Criteria/Description</th>
<th>Old Grading</th>
<th>New Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>No evidence of rejection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Focal perivascular and/or interstitial infiltrate without myocyte damage (Mild-Focal)</td>
<td>1A</td>
<td>1R (Mild)</td>
</tr>
<tr>
<td>Diffuse infiltrate without myocyte damage</td>
<td>1B</td>
<td>2 (Moderate-Focal)</td>
</tr>
<tr>
<td>One focus of infiltrate with myocyte damage</td>
<td>2</td>
<td>3A (Moderate-Multifocal)</td>
</tr>
<tr>
<td>Multifocal infiltrate with myocyte damage</td>
<td>3A</td>
<td>2R (Moderate)</td>
</tr>
<tr>
<td>Diffuse infiltrate with myocyte damage</td>
<td>3B</td>
<td>3R (Severe)</td>
</tr>
<tr>
<td>Diffuse polymorphous infiltrate with extensive myocyte damage ± edema ± hemorrhage ± vasculitis</td>
<td>4</td>
<td>(Severe)</td>
</tr>
</tbody>
</table>

Treatment Required

Data from Billingham et al16 and Stewart et al.17
invasive heart biopsy to detect rejection. With AlloMap molecular expression testing (XDx, Brisbane, CA) we have a simple blood test that can actually tell us whether patients are at risk of rejection. We also now have the ImmunKnow test (Cylex Inc., Columbia, MD) that tells us how immunoresponsive patients are by measuring the amount of adenosine triphosphate (ATP) that T cells make. Activated T cells make a lot of ATP, which means that immunosuppressed patients have lower amounts of ATP. This is most often a good thing, but too much immunosuppression increases the risk of infection. Thus, low test results indicate overimmunosuppression, mid-level results indicate proper immunosuppression, and higher results suggest that there is not enough immunosuppression on board. Studies in kidney and liver transplantation, and early data in heart transplant, suggest we can use this test to measure immunoresponsiveness rather than simply measuring blood levels of our immunosuppressive agents.

**Dr Russell:** In our hands, the ImmunKnow test seems to be better at identifying overimmunosuppressed patients at increased risk for infection more than underimmunosuppressed patients at increased risk for rejection. Have you detected both the highs and the lows?

**Dr Kobashigawa:** In our first experience with 46 patients, we did pick up patients who developed rejection. They had a mean ImmunKnow score of approximately 400. Those with scores below 200 or so had more infection. In our experience, the heart transplant patients should probably be between 200 and 350.

**Dr Rogers:** I like this movement toward a more personalized approach to manipulating immunosuppressive therapy. We have been lulled into thinking that immunosuppressive drug levels provide a comprehensive view of the immunosuppressed state.

**Dr Eisen:** Yes, these types of tests may eventually help us stave off late problems associated with overimmunosuppression, such as malignancy.

**Dr Conte:** Have these tests already impacted the scheduling or frequency of your endomyocardial biopsies?

**Dr Eisen:** Yes, in our program the AlloMap test, which was evaluated in the CARGO study, has resulted in a decline in biopsies beyond 6 months. For patients below a certain AlloMap threshold and without evidence of allograft dysfunction, we have elimi-
nated biopsies. We still need more information on the test's usefulness in the first 6 months before we start eliminating early biopsies.

**Dr Kobashigawa:** At this point, we have shown these 2 tests can detect risk for rejection, but we need studies to see if they can actually predict rejection and guide therapy to improve outcomes. The IMAGE (Invasive Monitoring Attenuation Through Gene Expression) trial with AlloMap is doing that now. Also, we should keep in mind that these tests are complementary, with the ImmunKnow test gauging the state of immunosuppression and the AlloMap test detecting low risk for rejection.

**Dr Rogers:** How often do you test tissue for markers of antibody-mediated rejection? And what are you actually staining for?

**Dr Eisen:** In our program, we do not routinely check for C4d or other complement markers unless we suspect antibody-mediated rejection due to, for example, graft dysfunction. And then only in the absence of significant cellular rejection would we initiate therapies for antibody-mediated rejection—therapies such as plasmapheresis to remove the antibodies, an anti-B cell agent like rituximab, or more traditional agents like cyclophosphamide or intravenous immunoglobulin (IVIG) to inhibit B-cell function.

**Dr Kobashigawa:** A National Institutes of Health Consensus Conference on humoral rejection in all organ transplantation established 4 criteria for the diagnosis of humoral rejection: (1) H&E (hematoxylin and eosin) for general staining and CD68 staining for intravascular macrophages in the capillaries; (2) immunohistochemistry staining to highlight antibodies on the endothelial cells; (3) circulating antibodies; and (4) cardiac or organ dysfunction. Of course, most of us do not employ all these specialty stains. Instead, we usually consider the diagnosis of antibody-mediated rejection when we see graft dysfunction but cannot find evidence of cellular rejection. In terms of the therapies, the most common are plasmapheresis and IVIG but we are seeing more use of rituximab and even antithymocyte globulin, which blocks not only T cells, but B cells and potentially plasma cells.

**Dr Rogers:** Do you prospectively screen all your patients for antibody-mediated rejection?

**Dr Kobashigawa:** We automatically look at the H&Es, and our pathologist also stains for CD68, CD3, CD20, and CD34. We also do C4d staining for complement but that is not absolutely necessary to make a diagnosis for antibody-mediated rejection.

**Dr Russell:** If a patient is doing well and some of those stains come back positive, do you just monitor the patient or do you change therapy?

**Dr Kobashigawa:** We actually studied patients with asymptomatic humoral rejection on the biopsy—with normal ejection fraction by echo—and found that these patients developed more CAV at 5 years compared to patients without humoral rejection. But we still do not have the data to tell us if we should treat these patients with agents like IVIG or high-dose corticosteroids empirically. We need more studies.

**Dr Eisen:** We should note that a CARGO II trial in Europe is now evaluating whether or not gene expression testing can detect antibody-mediated rejection.

### REFERENCES


